Supplementary Information

Divergent Binding Modes Direct Functional Modulation: Toward Next-Generation Ionic Liquids for Enzyme Stabilization and Biocatalysis

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1. MATERIALS AND METHODS

1.1 Materials: Commercially lyophilized powder of α-Chymotrypsin was procured from Sigma Aldrich. All chemicals (solvents, reagents, and chemicals) were purchased from the best-known local chemical suppliers and used without further purification. Solvents were distilled and dried prior to use. FTIR spectra were recorded on a Perkin-Elmer FT-IR Spectrum BX system and were reported in wave numbers (cm^{-1}). On the other hand, Mass spectra were recorded on Shimadzu LC-MS.

1.2 Sample preparation:

In this work, a 10 mM potassium phosphate buffer of pH 6.5, was prepared in double-distilled deionized water and was used to prepare all the samples. The protein concentration was fixed at 0.1 mg/mL, and the ILs concentration was fixed at 220 μM for all the studies.

2. INSTRUMENTATION OR METHODS

2.1 UV-Visible Spectroscopy:

The UV-vis spectroscopic studies were recorded on a JASCO (model V-650) UV-Vis spectrophotometer. The slit-width for the experiment was kept at 5 nm. Titration was carried out by adding requisite amounts of ILs into solutions of CHT in buffered medium (pH 6.5). The spectroscopic measurements were performed in triplicate (n = 3) under identical experimental conditions, and the results are expressed as mean ± standard deviation (SD).

2.2 Steady State Fluorescence:

The Steady State Fluorescence spectroscopic studies were recorded on a Shimadzu (RF-6000) Fluorescence spectrophotometer. Titration was carried out by adding requisite amounts of ILs into solutions of CHT in buffered medium (pH 6.5). the spectra were obtained at excitation wavelength of 280 nm and the slit width of both excitation and emission were set at 5 nm. The spectroscopic measurements were performed in triplicate (n = 3) under identical experimental conditions, and the results are expressed as mean ± standard deviation (SD).

2.3 Dynamic Light Scattering Measurements for Chymotrypsin in the Presence of ILs:

The hydrodynamic diameter (d_H) of CHT in phosphate buffer medium (pH 6.5) with ILs were obtained via dynamic light scattering (DLS) measurement, which were carried out by using a Zetasizer Nano S (Malvern Instruments) at 25 °C.

2.4 CHT Enzymatic Activity:

Time-dependent enzymatic activity of chymotrypsin (CHT) was monitored using a steady-state fluorescence spectrophotometer. A stock solution of the fluorogenic substrate PYR–TYR (10 μM) was prepared in 10 mM sodium phosphate buffer (pH 7.4). The activity of the CHT–IL composite was assessed by recording fluorescence changes over a time course of 0–18 min under identical experimental conditions.

2.5 Fluorescence Decay Experiment:

Fluorescence lifetime values were measured by using a time-correlated single photon counting fluorimeter (Horiba Jobin Yvon). The system was excited with nano LED of Horiba - Jobin Yvon with a pulse duration of 1.2 ns. Average fluorescence lifetimes (τ_{av}) for the exponential iterative fitting were calculated from the decay times (τ_i) and the relative amplitudes (a_i) using the following relation

$\tau_{av} = (a_1\tau_1^2 + a_2\tau_2^2 + a_3\tau_3^2)/ (a_1\tau_1 + a_2\tau_2 + a_3\tau_3)$

Where a_1, a_2, and a_3 are the relative amplitudes and τ_1, τ_2, and τ_3 are the lifetime values, respectively. For data fitting, a DAS6 analysis software version 6.2 was used.

2.6 ^{1}H & ^{13}C-NMR studies:

The synthesized ionic liquids (ILs) were characterized by using Bruker avance 500 MHz NMR instrument, in which chemical shifts reported in parts per million using as standard solvent signal ($CDCl_3$: ^{1}H, 7.26 ppm; ^{13}C, 77.16 ppm).

2.7 Stern–Volmer Quenching and Binding Constant Analysis:

The fluorescence quenching of chymotrypsin (CHT) by ionic liquids (ILs) was analyzed using the modified Stern–Volmer equation-

$$\text{Log}[(F_0-F)/F] = \log K_{SV} + n \log[IL]$$

where F_0 and F are the fluorescence intensities of CHT in the absence and presence of quencher (IL), respectively, [IL] is the quencher concentration, and K_{SV} is the Stern-Volmer quenching constant. n is the number of binding sites. The plot of log [(F0-F)/F] versus log[IL] yields K_{SV} from the intercept and n from the slope.

2.8 Thermodynamic parameters calculation:

The thermodynamic parameters (ΔH, ΔS, and ΔG) were calculated from fluorescence data obtained at two different temperatures (298 K and 323 K). The binding constants (K_1 and K_2) determined at these temperatures were used in the two-point van't Hoff equation:

$$\ln\left(\frac{K_2}{K_1}\right) = -\frac{\Delta H}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

From this, ΔH was obtained, and ΔS was determined using:

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

The Gibbs free energy change (ΔG) for each temperature was calculated using:

$$\Delta G = -\mathrm{RT}\ln K$$

3. DOCKING STUDIES

3.1 Receptor and ligand preparation and visualization:

The coordinates of the receptor Chymotrypsin (PDB ID: 4cha) was extracted from the crystallographic structures as available in RCSB with a resolution of 1.68 Å. Input files are prepared using Autodock tools 1.5.7. Nonpolar hydrogens are merged with the parent atom in both the receptors and the ligands, and Gasteiger charges are assigned to the ligands. Docking simulations were performed using Auto Dock Vina with an exhaustiveness parameter of 8 to ensure an adequate search of the conformational space. The resulting complexes were analyzed and visualized using Discovery Studio Visualizer.

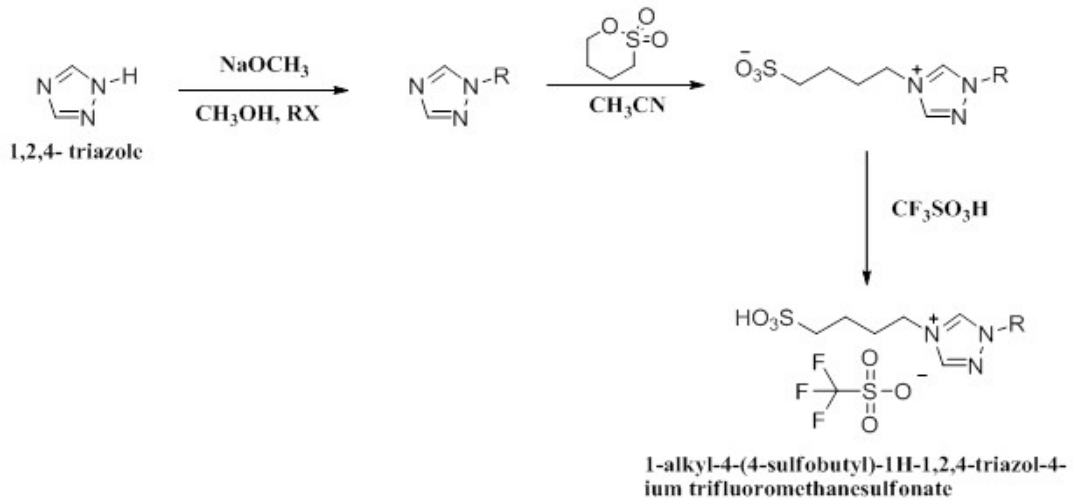
4. SYNTHESIS AND CHARACTERIZATION

4.1 Synthesis of ILs:

N-1 substituted 1,2,4-triazolium-based ILs (1-alkyl-4-(4-sulfobutyl)-1H-1,2,4-triazol-4-ium trifluoromethanesulfonate) were synthesized by N-1 alkylation followed by atom neutralization reaction through zwitterion intermediate (scheme 1). [1-3]

Briefly to a solution of 1,2,4-triazole (16.4 mmol) in dry methanol (15 ml), dry methanolic solution of sodium methoxide (16.8 mmol) was added. The mixture was left to stir for 30 min at room temperature, and then 1-bromo-alkane (16.56 mmol) was added dropwise. The reactant mixture was allowed to stir for 4 h followed by a reflux condenser overnight at 70 °C. After completion, the methanol was removed in a rotary evaporator and the halide impurity was removed by with water and ethyl acetate mixture. The crude product was purified through column chromatography in neutral alumina. The resulting solution was then placed in a high vacuum, affording a colorless liquid (89% yield). Then in a double-necked round-bottomed flask, 1-4-butane sultone (14.4 mmol) was added dropwise to 1-alkyl-1,2,4- triazole (14.4

mmol) at 0ºC. The resulting solution was allowed to stir at room temperature until a solid was obtained. Further, the reaction mixture was washed with toluene followed by diethyl ether in order to remove unreacted starting materials. The crude product was dried under reduced pressure to get the zwitterionic compound. This zwitterionic compound was further allowed to react with trifluoromethane sulfonic acid (14.5 mmol) at 0 ºC. Resulting solution was stirred at 60 ºC until a thick liquid formed. Obtained thick liquid was washed with diethyl ether to remove the excess trifluoromethane sulfonic acid. A light yellow color liquid was obtained, which was the desired IL, and this was placed under high vacuum (85% yield).



Scheme 1 Synthetic scheme of 1,2,4-triazolium-based ionic liquids.

4.2 Characterization of ILs:

IL-1: *1-pentyl-4-(4-sulfobutyl)-1H-1,2,4-triazol-4-ium trifluoromethanesulfonate* ([PtTzm][CF_3SO_3]): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.77 (s, 1 H), δ 8.58 (s, 1 H), 4.55 (t, J = 5.5 Hz, 2H), 4.40 (t, J = 7.5 Hz, 2H), 3.16 (t, 6.25 Hz, 2H), 2.25 (m, 2H), 1.97 (m, 2 H), 1.85 (m, 2 H), 1.34 (m, 4 H), 0.90 (t, J = 7.0 Hz 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ 142.7, 140.9, 123.9, 121.4, 118.9, 116.4, 74.1, 52.7, 48.4, 28.6, 28.2, 23.7, 22.9, 22.0, 13.8. HRMS (ESI+) *m/z* for $[M]^+$ calcd 276.1382, found 276.1377.

IL-2:*1-butyl-4-(4-sulfobutyl)-1H-1,2,4-triazol-4-iumtrifluoromethanesulfonate* ([BtTzm][CF_3SO_3]): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.74 (s, 1 H), δ 8.56 (s, 1 H), 4.54 (t, J =

5.0 Hz, 2H), 4.42(t, 7.25 Hz, 2H), 3.15 (t, 6.25 HZ, 2H), 2.24 (m, 2H), 1.96 (m, 2 H), 1.84 (m, 2 H), 1.38 (m, 2 H), 0.97 (t, 7.5 HZ, 3H).^{13}C NMR (125 MHz, $CDCl_3$) δ 142.7, 140.9, 124.0, 121.5, 119.0, 116.4, 74.1, 52.5, 48.4, 30.8, 23.7, 23.0, 19.4, 13.2.

IL-3: *1-isopentyl-4-(4-sulfobutyl)-1H-1,2,4-triazol-4-ium trifluoromethanesulfonate* ([IpTzm][CF_3SO_3]): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 1 H), δ 8.55 (s, 1 H), 4.55 (t, J = 5.0 Hz, 2H), 4.43 (t, 7.5 HZ, 2H), 3.16 (t, 6.25 Hz, 2H), 2.25 (m, 2H), 1.87 (m, 4 H), 1.64 (m, 1 H), 0.98 (d, 6 H).^{13}C NMR (125 MHz, $CDCl_3$) δ 142.6, 140.8, 124.0, 121.5, 118.9, 116.4, 74.0, 51.3, 48.4, 37.6, 25.6, 23.7, 23.0, 22.0, 19.6. HRMS (ESI+) *m/z* for [M]+ calcd 276.1382, found 276.1373.

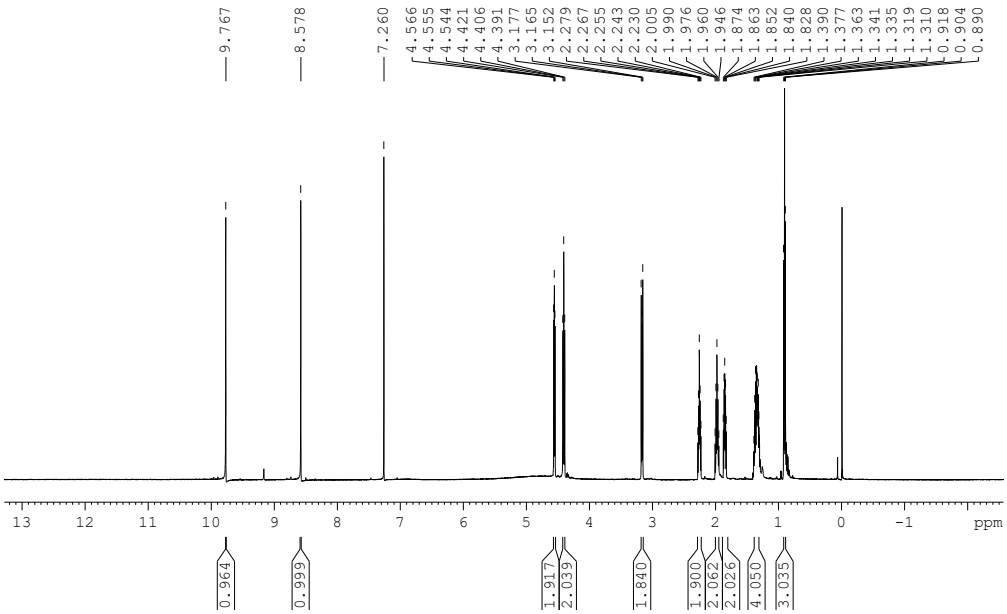


Figure S1. ^{1}H NMR spectra of IL1

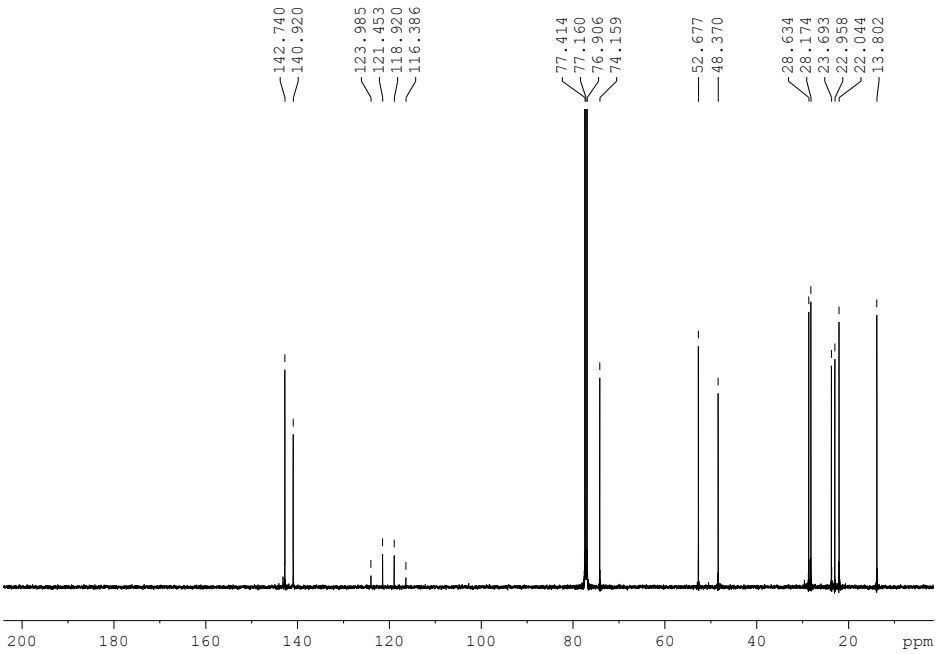


Figure S2. ^{13}C NMR spectra of IL1

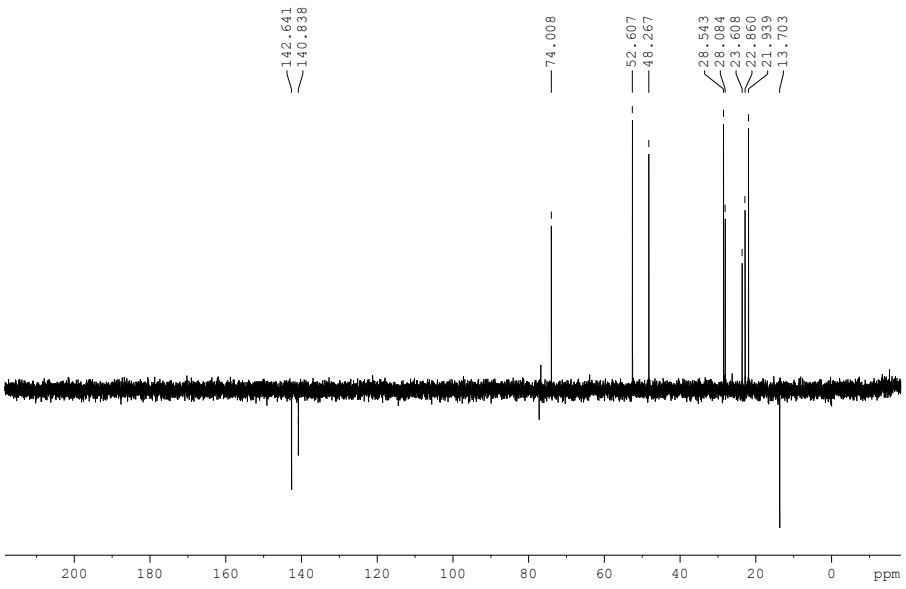


Figure S3. DEPT NMR spectra of IL1

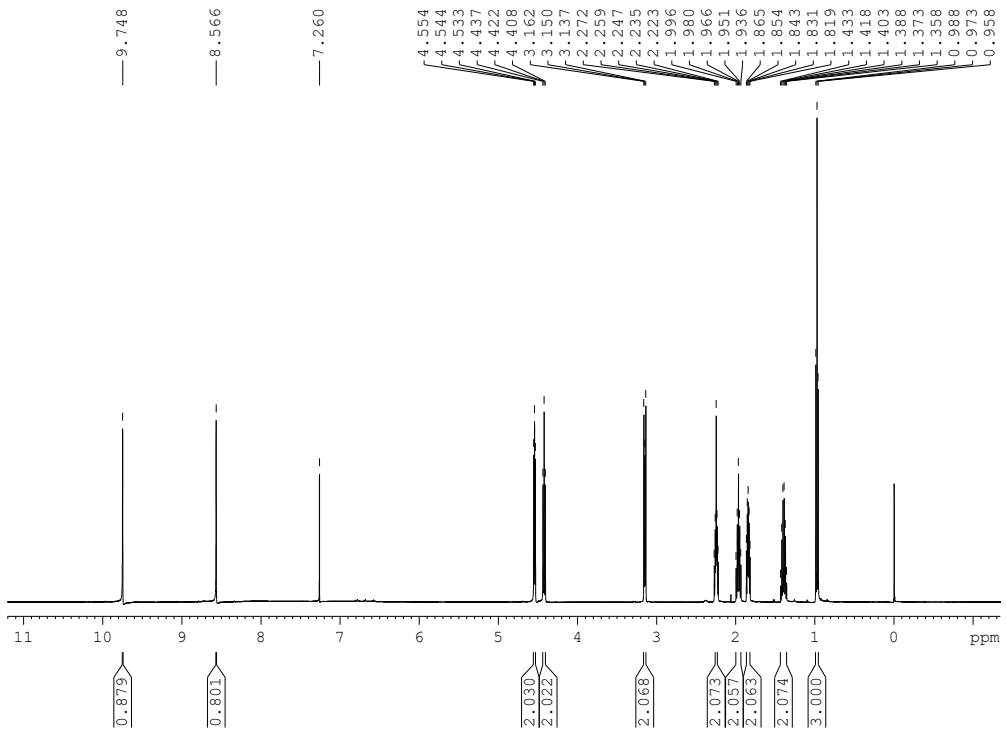


Figure S4. ^{1}H NMR of IL2

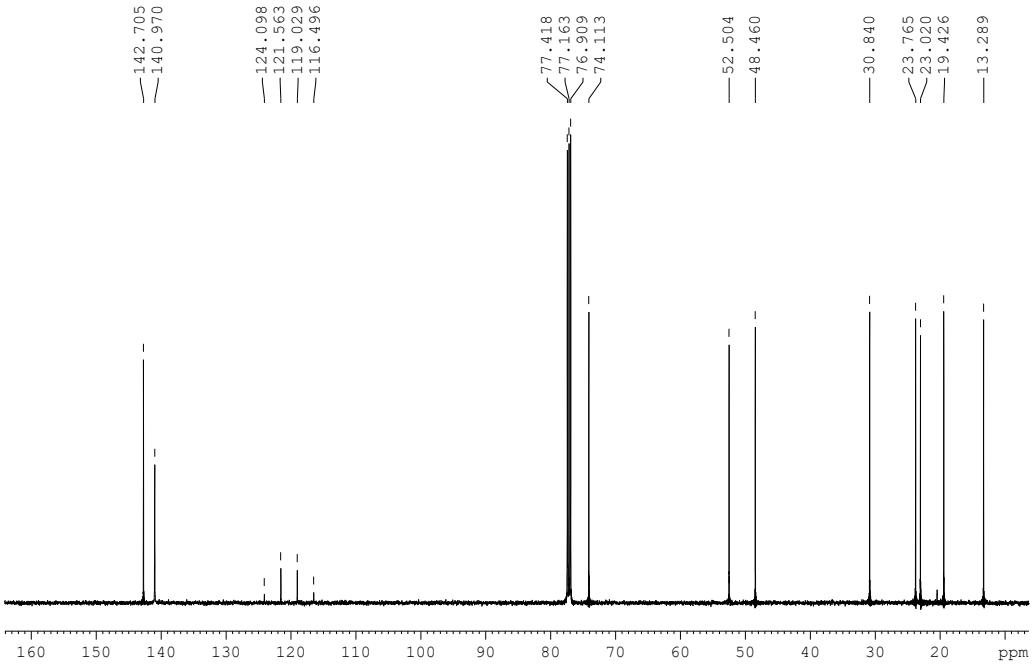


Figure S5. ^{13}C NMR of IL2

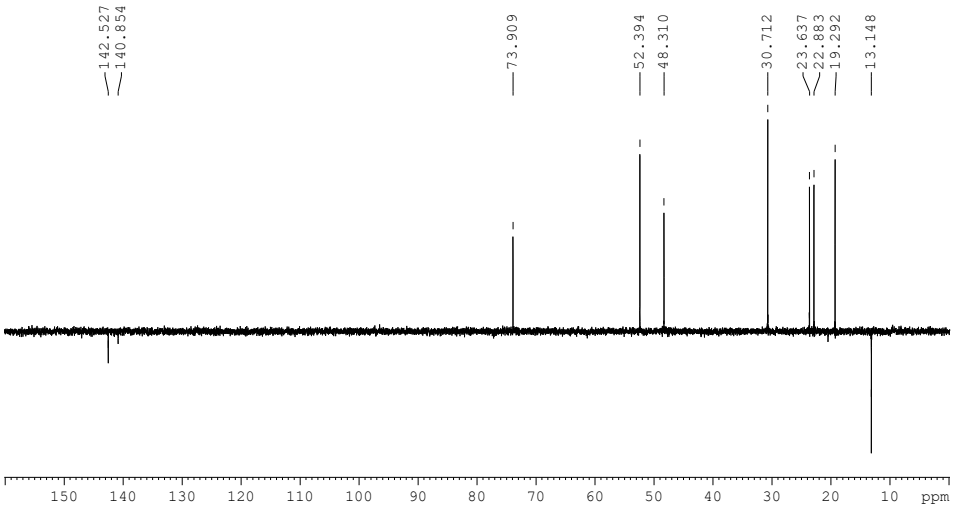


Figure S6. DEPT NMR of IL2

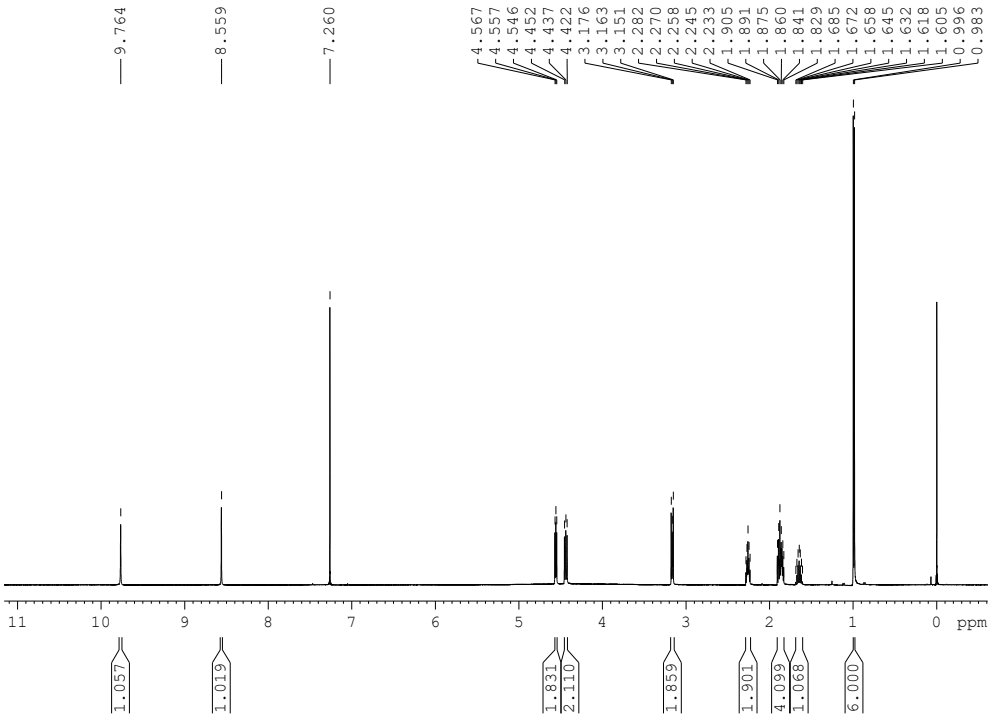


Figure S7. ^{1}H NMR of IL3

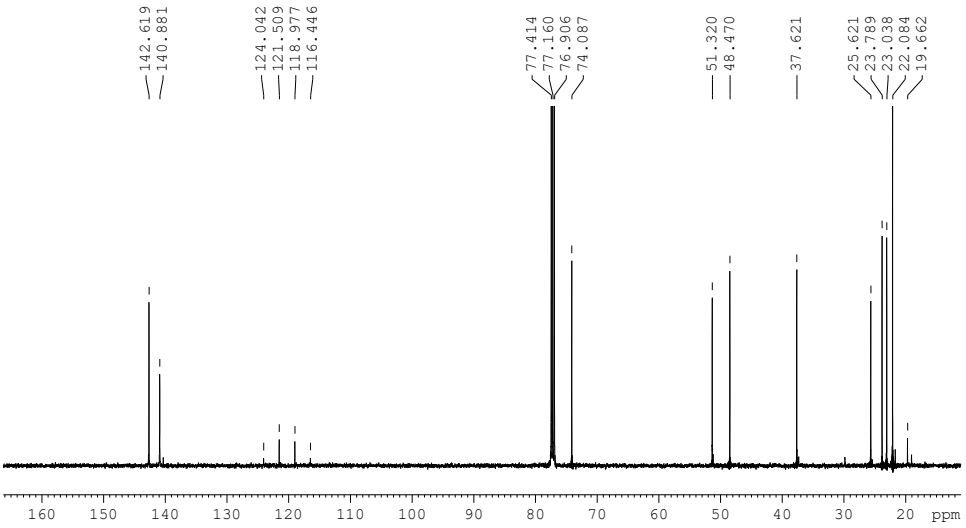


Figure S8. ^{13}C NMR of IL3

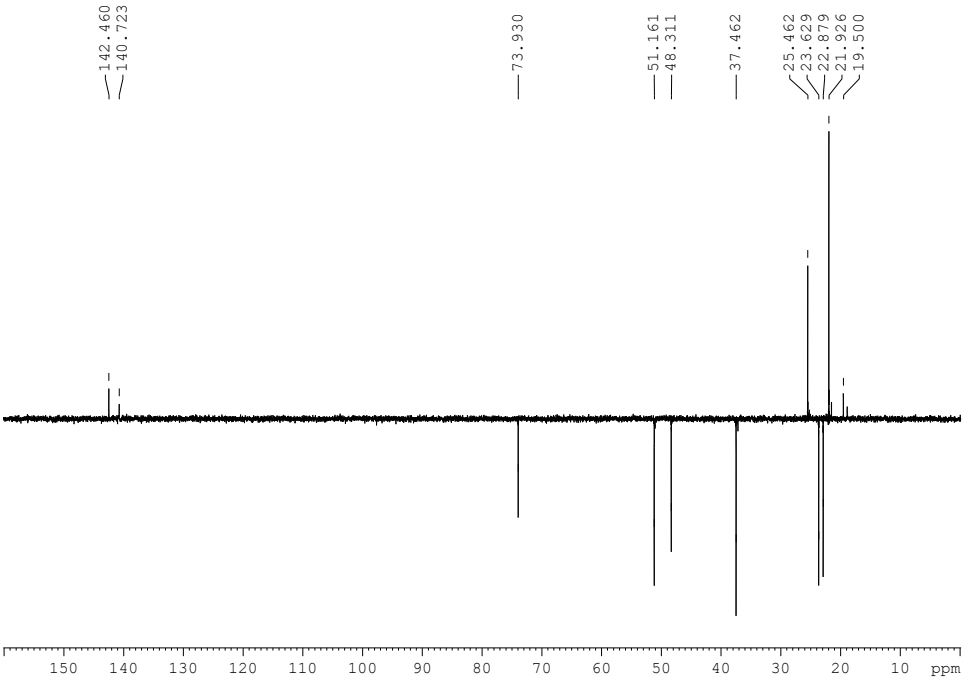


Figure S9. DEPT NMR of IL3

5. ADDITIONAL SPECTROSCOPIC DATA

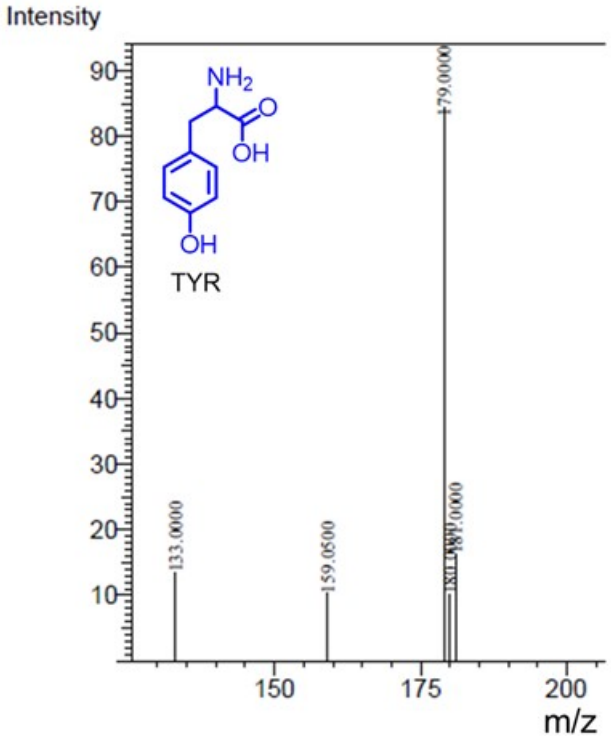


Figure S10. LC-MS data of the catalysed product of PYR-TYR (10 μM) in the presence of CHT.IL composite.

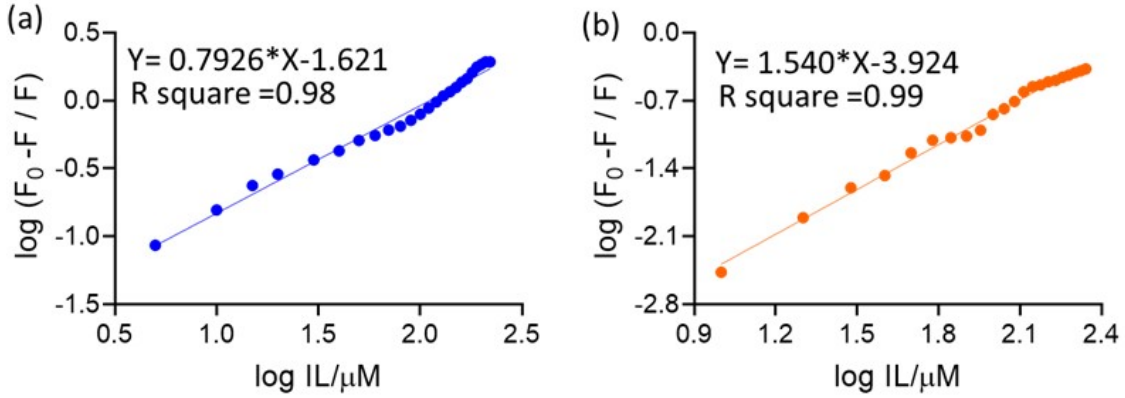


Figure S11. Modified Stern-Volmer plots of log[(F_0-F)/F] versus log[IL] for determining the binding constant of the CHT-IL1 interaction at (a) 25 °C and (b) 50 °C temperature in buffer medium (pH 6.5) derived from fluorescence titration data.

Table S1: Fluorescence lifetimes of CHT in buffer medium at pH 6.5 with and without ILs.

System	a_1	a_2	a_3	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	τ_{av} (ns)	chi-square (χ^2)
CHT (0.1 mg/mL) λ_{ex} = 280 nm, λ_{em} = 335 nm	0.09	0.86	0.05	1.18	0.080	3.59	2.18	0.97

CHT (0.1 mg/mL) + IL1 (220 µM) λ_{ex} = 280 nm, λ_{em} = 335 nm	0.93	0.05	0.01	0.17	2.78	5.5	2.08	1.0
CHT (0.1 mg/mL) + IL3 (220 µM) λ_{ex} = 280 nm, λ_{em} = 335 nm	0.14	0.81	0.05	1.26	0.13	3.86	2.05	1.12
CHT (0.1 mg/mL) λ_{ex} = 280 nm, λ_{em} = 405 nm	0.28	0.01	0.71	4.60	37.43	1.57	7.8	1.23
CHT (0.1 mg/mL) + IL3 (220 µM) λ_{ex} = 280 nm, λ_{em} = 405 nm	0.21	0.01	0.78	5.54	57.29	1.83	13.5	1.17

Types of IL	**Enzyme Studied**	**Key Interaction Mechanism**	**Effect on stability**	**Effect on Catalytic Activity**	**References**
Imidazolium-based ([Bmim][BF_4])	Trypsin (free and immobilized)	Predominantly hydrophobic and electrostatic interactions between the [Bmim]$^+$ cation and the enzyme surface	At relatively low [Bmim][BF_4] content, trypsin keeps its secondary structure reasonably well, but higher IL fractions lead to structural perturbation and loss of stability.	Enzymatic activity is largely maintained only at low IL concentrations	Journal of Molecular Liquids, 171, 16-22.
Imidazolium-based ILs	Alpha-Chymotrypsin	Hydrophobic and electrostatic interactions between [C_4mim]-type cations and aromatic/charged residues	Moderate stabilization at optimized IL composition; high IL content promotes unfolding/aggregation.	Often decreases activity at higher IL fraction	International journal of biological macromolecules 51.4 (2012): 555-560.
Imidazolium/other ILs	Multiple enzymes (lipases, proteases, oxidases)	ILs act as tunable solvents or co-solvents; cation/anion structure controls protein compatibility.	Certain ILs improve storage and thermal stability, especially hydrophilic, low-toxicity systems.	ILs can increase reaction rates and in biocatalytic processes	RSC Adv., 2025, 15, 45744
Imidazolium-based ([C_4mim][Cl])	Alpha-Chymotrypsin	Strong cation-π and hydrogen-bonding interactions	Moderate increase in structural stability around 40 °C, but tendency toward partial unfolding and loss of native packing at higher IL	Approx. 20% decrease in catalytic activity, attributed to increased conformational rigidity	Journal of Biomolecular Structure and Dynamics, 32(8), 1263–1273

			concentration		
Phosphonium-based (e.g., $[P_{66614}]$ $[NTf_2]$)	Lipase	Strong hydrophobic and ion-pair interactions with support and enzyme microenvironment.	Enhanced thermal/operational stability; IL-treated biocatalysts are reusable over many cycles	Activity boosts up to 2-3-fold compared to IL-free immobilized enzyme.	ACS Sustainable Chem. Eng. 2019, 7, 15648−15659

Table S2: Comparison of reported imidazolium and phosphonium-based ionic liquids (ILs) with various proteolytic enzymes.

Types of IL	Enzyme Studied	Key Interaction Mechanism	Effect on stability	Effect on Catalytic Activity	References
Imidazolium-based ionic liquids ([Bmim][I])	α-Chymotrypsin	Combination of electrostatic, cation–π, and hydrogen-bonding interactions between the imidazolium cation/anion pair and surface residues	Certain IL compositions enhance thermal stability and resistance to unfolding	Moderate IL contents often maintain or slightly reduce activity, while higher fractions can suppress turnover due to restricted dynamics and active-site perturbation.	Phys. Chem. Chem. Phys., 2015, 17, 184
Imidazolium-based ($[C_8mim][Cl]$)	Papain	Hydrophobic insertion of the octyl chain into nonpolar regions near the active-site cleft, combined with electrostatic and hydrogen-bonding contacts between the imidazolium headgroup and surface residues.	Moderate enhancement of structural stability around 45 °C, with improved resistance to thermal unfolding	Proteolytic activity remains high at low–moderate IL concentration, with roughly 80–90% of native activity retained under conditions where structure is stabilized.	Applied biochemistry and biotechnology, 168(3), 592-603
Imidazolium, phosphonium, cholinium, amino acid ILs	Proteases, oxidoreductases, therapeutic proteins,	Protein–IL compatibility is “bispecific”: stability depends on both IL ion structure (hydrophobicity, hydrogen-bonding ability, Hofmeister character) and the protein’s surface	ILs can either strongly stabilize or destabilize proteins; appropriately designed ILs	Catalytic activity can be maintained or enhanced when ILs preserve native-like hydration and dynamics	Chem. Rev. 2024, 124, 3037–3084.
Triazolium-based ZIL (this work)	Chymotrypsin	Balanced electrostatic and hydrophobic interactions; charge-transfer stabilization	Enhanced thermal stability; reduced unfolding	Slightly improved catalytic rate (~28%)	This work

References:

1. D. Singh, R.L. Gardas, J. Phys. Chem. B 120 (2016) 4834–4842.
2. D. Singh, R.L. Gardas, J. Mol. Liq. 250 (2018) 477–484.
3. D. Singh, G. Sharma, R.L. Gardas, ChemistrySelect 2 (2017) 3943–3947.